J. Cell Sci. 56, 461–470 (1982)
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INFILTRATION OF LYMPHOSARCOMA CELLS INTO HEPATOCYTE CULTURES: INHIBITION BY UNIVALENT ANTIBODIES AGAINST LIVER PLASMA MEMBRANES AND LYMPHOSARCOMA CELLS

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SUMMARY

The number of murine MB6A lymphosarcoma cells that infiltrated rat hepatocyte cultures was found to be diminished after treatment of the lymphosarcoma cells with univalent antibodies raised against these tumour cells (anti-MB6A Fab), and also after treatment of the hepatocyte cultures with univalent antibodies directed against rat liver plasma membranes (anti-LPM Fab).

The inhibition of infiltration by anti-MB6A Fab and an anti-LPM Fab raised against sinusoidal face-enriched membranes could be entirely attributed to their interference with adhesion of MB6A cells to the exposed surface of the hepatocytes, because infiltration of the adherent cells was not inhibited. Anti-LPM Fab raised against contiguous face-containing LPM, on the other hand, inhibited the adhesion to the exposed surface and the subsequent infiltration of adherent cells. These observations suggest that specific membrane constituents of both MB6A cells and hepatocytes take part in liver infiltration, and that there may be two different hepatocyte components involved, one mediating adhesion to the exposed surface and the other taking part in the infiltration process proper.

INTRODUCTION

Previously (Roos, Van de Pavert & Middelkoop, 1981), we described the infiltration of MB6A lymphosarcoma cells into cultures of isolated adult hepatocytes. We use these cultures as a model for the infiltration of MB6A cells into the intact liver during the formation of hepatic metastases (Dingemans, 1973; Roos, Dingemans, Van de Pavert & Van den Bergh Weerman, 1977). After adhesion to the hepatocytes the MB6A cells rapidly infiltrated between and under the liver cells and accumulated there at interhepatocyte boundaries. After 24 h, virtually all tumour cells were located within the cultures.

We assumed that specific adhesion molecules were involved in this process. Such molecules that mediate mutual adhesion of cells and their adhesion to extracellular substrates, have been identified on several different cell types (Takeichi, 1977; Thiery, Brackenbury, Rutishauser & Edelman, 1977; Müller & Gerisch, 1978; Wylie, Damsky & Ruck, 1979; Bertolotti, Rutishauser & Edelman, 1980; Ocklind, Rubin &

Öbrink, 1980; Nielsen, Pitts, Grady & McGuire, 1981). To explain the accumulation of lymphosarcoma cells within the cultures, we assumed further that the contiguous surface of hepatocytes is more adhesive for lymphosarcoma cells than the exposed surface, causing the tumour cells to be arrested at the contiguous surface. This increased adhesiveness could be due to a higher density of the same adhesion molecule or to the presence of a different molecule for which these tumour cells have a higher affinity.

To establish whether these assumptions are correct, we used an immunological method, which has been successfully applied to the identification of several different adhesion molecules on various cell types (Rosen, Haywood & Barondes, 1976; Brackenbury, Thiery, Rutishauser & Edelman, 1977; Thiery *et al.* 1977; Müller & Gerisch, 1978; Öbrink & Ocklind, 1978; Urushihara, Ozaki & Takeichi, 1979; Wylie *et al.* 1979; Bertolotti *et al.* 1980; Ocklind *et al.* 1980), including embryonal (Bertolotti *et al.* 1980) and adult (Öbrink & Ocklind, 1978; Ocklind *et al.* 1980; Nielsen *et al.* 1981) hepatocytes. This method is based on inhibition of adhesion by Fab fragments of immunoglobulins raised against whole cells or plasma membranes. Pretreatment of the Fab fragments with the isolated adhesion molecule neutralizes the inhibiting activity. Thus, using a multispecific antiserum, adhesion molecules can be identified by pretreatment of the Fab fragments with purified membrane components.

In this report we present data on the inhibition of both adhesion and infiltration of lymphosarcoma cells into hepatocyte cultures, by Fab fragments prepared from antibodies raised against lymphosarcoma cells or against liver plasma membrane preparations, containing either contiguous face membranes or sinusoidal face-enriched membranes. The effects of the different antisera point to the existence of at least one, but possibly two, MB6A adhesion molecule(s) binding to two different components on liver cells: one on the exposed surface mediating the initial adhesion of MB6A cells to the cultures, and another on the contiguous surface involved in infiltration.

MATERIALS AND METHODS

Hepatocyte isolation and culture

Rat hepatocytes were isolated as described (Roos *et al.* 1981), and cultured using the modified method described by Roos & Van de Pavert (1982).

Tumour cells

Murine MB6A ascites lymphosarcoma cells were maintained and harvested as described before (Roos *et al.* 1981).

Preparation of liver plasma membranes (LPM)

Contiguous face-containing LPM (CLPM) were prepared according to Emmelot, Bos, Van Hoeven & Van Blitterswijk (1974), omitting overnight washing in Ca^{2+}-free phosphate-buffered saline (PBS). Antisera kindly provided by Dr B. Öbrink were raised against CLPM, prepared

according to Ray (1970), and against liver plasma membrane preparations enriched in sinusoidal membranes (SLPM), isolated according to Wisher & Evans (1975).

Antisera and Fab fragments

Rabbit antisera were produced by intramuscular injection of MB6A cells (10^7) or LPM (1 mg protein) in 1 ml PBS + 1 ml Freund's complete adjuvant. Booster injections were given monthly with the same amount of antigen + Freund's incomplete adjuvant. Blood was collected 1 week after a booster. The antisera used were obtained after 5–6 boosters with MB6A cells (antisera 1 and 2) and after 7 boosters with LPM (antisera 3 and 4). The obtained antisera reacted with the MB6A cells and cultured hepatocytes, respectively, as was demonstrated with fluorescein-labelled goat anti-rabbit immunoglobulin G (IgG).

Immunoglobulins were isolated from the antisera using a protein A-Sepharose column (Pharmacia). Fab fragments were prepared by treating the antibodies with papain (Boehringer; 1 mg per 50 mg protein in 0·03 M-NaCl, 0·02 M-Tris·HCl (pH 7·4), 0·05 M-cystein·HCl, 0·05 M-EDTA) at 37 °C for 15 h (Öbrink & Ocklind, 1978). The digest was dialysed (inactivating the papain by removal of cystein and EDTA) against PBS and the Fab fragments were separated from undigested IgG by passing the digest through a protein A–Sepharose column. Fab fragments were isolated by chromatography on Sephadex G-100 (Pharmacia).

Univalent antibodies against the asialoglycoprotein receptor of rat hepatocytes (Ashwell & Morell, 1974) were prepared from a goat antiserum to this receptor (a kind gift from Dr G. Ashwell; the serum contained approx. 1 mg specific antibodies per ml). This anti-serum reacted with our cultured hepatocytes, as shown by immunofluorescence with fluorescein-labelled rabbit anti-goat IgG. Univalent antibodies against the T200 antigen (Ledbetter & Herzenberg, 1979), present on MB6A cells as shown by immunofluorescence with fluorescein-labelled goat anti-rat IgG, were prepared from monoclonal antibodies against T200 antigen (from culture supernates of rat hybridoma cells given by Dr L. A. Herzenberg to Dr J. Hilgers of this Institute, ref. no. 30G12) by precipitation with $(NH_4)_2SO_4$ and purification on a Sephadex G-75 column, since these antibodies do not bind to protein A. Leupeptin (Protein Research Found., Japan; 20 μg/ml) was added to inhibit any residual papain activity, to which hepatocytes are very sensitive. All fluorescein-labelled antisera were obtained from Nordic, Tilburg, The Netherlands.

To absorb out inhibiting activity, Fab fragments were incubated with whole freshly isolated hepatocytes or MB6A cells for 30 min at 0 °C. Trasylol (Bayer, final concentration 150 Kallikrein Inactivator Units/ml) was added to inhibit the activity of proteases that might be released by dead cells during the absorption.

Assay of interaction and infiltration

After the hepatocytes had been in culture for 24 h, pieces of Petriperm (approx. 2 cm^2 with $2·5 \times 10^5$ hepatocytes) were cut and transferred to Petri dishes and covered with 0·2 ml incubation medium (DMEM + 20 mM-HEPES) containing 10^6 tumour cells. The cultures were incubated in 5 % CO_2/air at 37 °C for 4 h. Then the cells were fixed, dehydrated, scraped off the Petriperm pieces, pelleted and embedded in Epon. Sections of 1 μm, cut with an LKB Ultratome, were stained with 1 % toluidine blue and observed under the light microscope.

Treatment of MB6A cells and hepatocytes with Fab fragments

MB6A cells were treated with 0·1 mg anti-MB6A Fab per 10^6 cells, and the hepatocytes with 0·5 mg anti-LPM Fab per 10^6 cells, for 30 min at 37 °C. The antibodies remained present during the co-incubation of tumour cells and hepatocytes. Fab fragments prepared from pre-immune sera were used in control experiments.

Quantification of light microscopical observations

Observations were quantified as described by Roos & Van de Pavert (1982). In 1 μm sections through the Epon-embedded pellets, multiple culture fragments were present, cut at

various angles to the substrate. The substrate side was easily recognized as the extended straight side of the fragments. We counted:

N, *Hepatocyte nuclei* as a measure of the number of hepatocytes.

T, the total number of *interacting tumour cells*: all cells in contact with hepatocytes, including those that had infiltrated.

T_{Inf}, the number of *infiltrated tumour cells*: those cells that were completely surrounded, in the plane of the section, by hepatocytes or by hepatocytes and the substrate.

The results were expressed in the following parameters:

(1) *Interaction index*: $I = T/N$; i.e. the number of interacting cells per hepatocyte nucleus.

(2) *Infiltrated fraction*: T_{Inf}/T.

The extent of infiltration can be influenced by interference either with the adhesion to the exposed surface or with the migration of the adherent cells into the cultures. We assume that effects on these two processes are reflected in changes of the *interaction index* and the *infiltrated fraction*, respectively, as will be discussed.

In all experiments at least 150 hepatocyte nuclei were counted in each of duplicate cultures. Parameters presented are the average of the duplicates.

RESULTS

Antisera raised against lymphosarcoma cells

In Table 1 the results are given of an experiment in which the effect, on the interaction of MB6A cells with hepatocyte cultures, of Fab fragments of immunoglobulins raised against MB6A lymphosarcoma cells (anti-MB6A Fab) was measured. Antisera of two rabbits were used (Fab 1 and Fab 2). The interaction index was diminished.

Table 1. *Effect of anti-MB6A Fab on the interaction of MB6A cells with hepatocyte cultures: results of one experiment*

	Interaction index*	Infiltrated fraction†
Control	0·53 (100 %)	0·58 (100 %)
Fab 1	0·13 (25 %)	0·56 (97 %)
Fab 2	0·32 (60 %)	0·61 (105 %)

* Interacting cells per hepatocyte nucleus.
† Infiltrated cells divided by interacting cells.

Both the interaction index and the infiltrated fraction varied between experiments performed on different occasions, but there was little variation between duplicate cultures in one experiment. However, the results expressed as percentages of the control values were similar in all experiments. The averages of these percentages over four similar experiments are given in Table 2. Anti-MB6A Fab substantially reduced the total number of cells that infiltrated the cultures. This reduction can be attributed entirely to a reduction of the total number of interacting cells. Since the infiltrated fraction of the adherent cells was not reduced, the infiltration process proper was apparently not inhibited. Treatment of MB6A cells with 0·01 mg Fab instead of 0·1 mg per 10^6 cells resulted in a 30–40% loss of inhibition. Univalent antibodies against the T200 antigen, present on MB6A lymphosarcoma cells, had

Table 2. *Combined results of four experiments with anti-MB6A Fab similar to the one in Table 1: average of percentages of controls*

	Interaction index (%)	Infiltrated fraction (%)
Controls	100	100
Fab 1	32 (±14)*	100 (±9)
Fab 2	55 (±10)	75 (±21)

* Standard deviation.

Table 3. *Effect of anti-MB6A Fab 2 absorbed with MB6A cells (0·375 mg Fab fragments with 20 × 10^6 cells)*

	Interaction index	Infiltrated fraction
Control	0·52 (100%)	0·46 (100%)
Fab 2	0·10 (19%)	0·79 (172%)
Absorbed Fab 2b	0·57 (110%)	0·49 (107%)

no effect. These antibodies could therefore be used as a control Fab, actually binding to the cells. The inhibiting activity of anti-MB6A antibodies was absorbed out by pretreatment of 0·375 mg Fab fragments with 20 × 10^6 MB6A cells (Table 3).

Antisera raised against liver plasma membranes (LPM)

In Table 4 the results are given of an experiment in which the effect, on the interaction between MB6A cells and hepatocyte cultures, was measured of Fab fragments of immunoglobulins raised against contiguous face-containing LPM (CLPM) prepared according to Emmelot *et al.* (1974) (Fab 3 and Fab 4), according to Ray (1970) (Fab 5), and against sinusoidal face-enriched membranes (SLPM), prepared according to Wisher & Evans (1975) (Fab 6). The latter two antisera were kindly provided by Dr B. Öbrink.

Three similar experiments were done. The percentages of control values were averaged and given in Table 5. Also the anti-liver Fab substantially reduced the

Table 4. *Effect of anti-LPM Fab on the interaction between MB6A cells and hepatocyte cultures: results of one experiment*

	Interaction index	Infiltrated fraction
Control	2·20 (100%)	0·51 (100%)
Fab 3[a]	1·51 (69%)	0·04 (8%)
Fab 4[a]	1·59 (72%)	0·19 (36%)
Fab 5[b]	0·67 (30%)	0·13 (25%)
Fab 6[c]	0·22 (10%)	0·55 (107%)

The Fab fragments were prepared from antisera raised against liver plasma membranes prepared according to: [a] Emmelot *et al.* (1974), [b] Ray (1970), [c] Wisher & Evans (1975; sinusoidal face-enriched LPM).

total number of cells that infiltrated. In the case of Fab 6 (against SLPM) this effect could be attributed entirely to reduction of the interaction index, since there was no effect on the infiltrated fraction. Apparently, this antiserum against sinusoidal membranes does not contain antibodies that can interfere with infiltration between hepatocytes. In contrast, Fabs 3, 4 and 5 (against contiguous face-containing LPM) reduced both the interaction index and the infiltrated fraction. Treatment of the hepatocytes with smaller amounts of Fab did not result in inhibition. Already, with 0·25 mg instead of 0·5 mg per 10^6 cells, no inhibition of the infiltrated fraction was observed.

Table 5. *Combined results of three experiments with anti-LPM Fab, similar to the one in Table 4: averages of percentages of controls*

	Interaction index (%)	Infiltrated fraction (%)
Controls	100	100
Fab 3	67 (±22)*	23 (±13)
Fab 4	70 (±20)	52 (±21)
Fab 5	38 (±15)	34 (±28)
Fab 6	15 (±5)	100 (±11)

* Standard deviation.

Table 6. *Effect of anti-LPM Fab 3 absorbed with hepatocytes (0·275 mg Fab fragments with 5×10^6 cells)*

	Interaction index	Infiltrated fraction
Control	2·01 (100%)	0·28 (100%)
Fab 3	1·40 (70%)	0·01 (4%)
Absorbed Fab 3	1·92 (96%)	0·32 (114%)

Univalent antibodies against the asialoglycoprotein receptor described by Ashwell & Morell (1974) had no effect, so that these antibodies could serve as control antibodies. The inhibiting activity of anti-LPM antibodies could be absorbed out by pretreatment of 0·275 mg Fab fragments with 5×10^6 freshly isolated hepatocytes (Table 6).

DISCUSSION

In this paper we present the first results of a study aimed at the identification of cell surface molecules involved in the interaction between lymphosarcoma cells and hepatocyte cultures. These molecules may also play a role in the infiltration process of these tumour cells in the intact liver.

We used an immunological method that is widely applied to the identification of adhesion components on different cell types (Rosen *et al.* 1976; Brackenbury *et al.* 1977; Takeichi, 1977; Thiery *et al.* 1977; Müller & Gerisch, 1978; Öbrink & Ocklind, 1978; Urushihara *et al.* 1979; Wylie *et al.* 1979; Bertolotti *et al.* 1980; Ocklind *et al.* 1980; Nielsen *et al.* 1981). In the first step of this method, adhesion

is inhibited with multispecific antibodies against membrane components. In subsequent experiments, the antibodies are pretreated with purified membrane components. If this neutralizes the inhibiting activity, the adhesion molecule can be identified. Univalent antibodies are used because divalent antibodies may agglutinate cells.

The first step of the method has now been performed successfully in our culture model. The number of infiltrating cells was substantially reduced in the presence of antibodies directed against both the tumour cells and the hepatocytes, which indicates that membrane components on both cells are involved in the infiltration process.

Decreased infiltration may be due either to reduction of adhesion to the exposed surface or to interference with the subsequent migration of adherent cells into the cultures. The two effects can be separated using the parameters 'interaction index' and 'infiltrated fraction', if it is assumed that: (1) infiltrated cells have first adhered to the exposed surface, so that the sum of infiltrated and non-infiltrated cells reflects total adhesion to this surface within the interval studied; and (2) the number of infiltrated cells is proportional to the number of adherent cells, so that the infiltrated fraction is independent of adhesion to the exposed surface. Data supporting these assumptions have been published (Roos & Van de Pavert, 1982). The reduction of infiltration by the antibodies against MB6A cells (Fabs 1 and 2) and against the sinusoidal liver membranes (Fab 6) may then be interpreted as being due to interference with adhesion to the exposed surface. The infiltration process proper of the adherent cells, however, was not affected, since the infiltrated fraction was not reduced. It appears likely that a component on MB6A cells, masked by the anti-MB6A Fab, and a hepatocyte component, masked by the anti-sinusoidal membrane Fab, are two complementary adhesion molecules, that mediate the attachment of the MB6A cells to the exposed surface of the hepatocytes.

In the case of antibodies against contiguous face-containing liver plasma membranes (Table 5, Fabs 3, 4 and 5) the effect was more complex. The three antibody preparations all reduced total interaction and thus presumably inhibited adhesion to the exposed surface, probably by masking the same component as Fab 6 did. In addition, they also inhibited the infiltration of the adherent cells, as expressed by the infiltrated fraction. In itself, this observation is compatible with only one adhesion molecule on liver cells being involved in both phenomena. However, when the effect of Fab 6 is taken into account, this explanation is highly unlikely. Since Fab 6 strongly inhibited adhesion, it should also have had at least some effect on the infiltration process, if the same hepatocyte component was involved. However, the infiltrated fraction was virtually identical to controls in all three experiments with Fab 6. The more likely explanation is, therefore, the presence of two different hepatocyte components, involved in the adhesion to the exposed surface and in infiltration, respectively. The fact that the anti-sinusoidal membrane antibodies do not seem to react with the second component indicates that it is not present at the sinusoidal, but exclusively at the interhepatocyte (contiguous) surface.

If both hepatocyte components did bind to the same MB6A surface molecule,

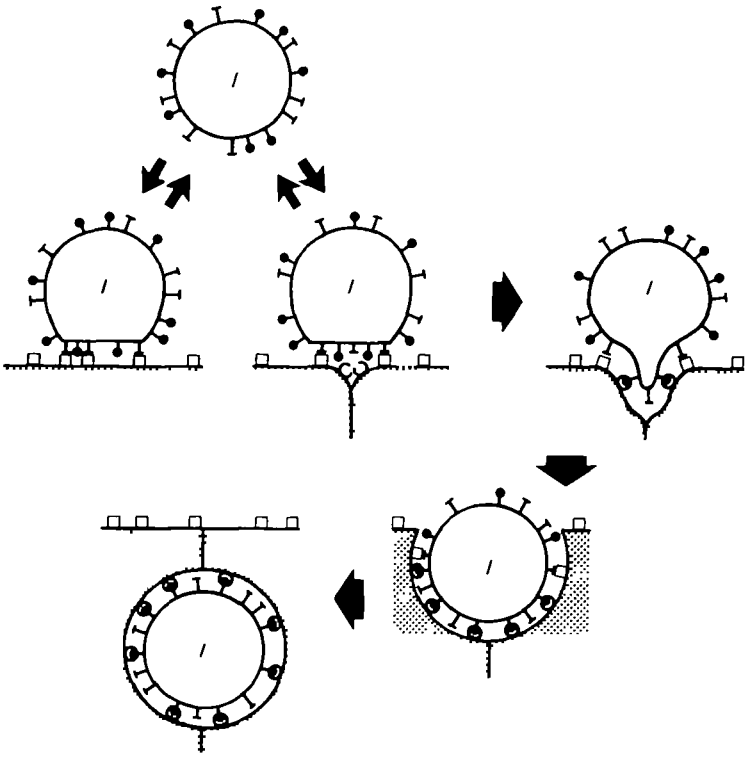

Fig. 1. A hypothetical model for infiltration of MB6A lymphosarcoma (*l*) cells into a hepatocyte (*h*) culture. MB6A cells bind reversibly to the exposed hepatocyte surface. This binding can be inhibited with anti-MB6A Fabs 1 and 2 (binding to ⊣) and with anti-LPM Fabs 3–6. MB6A component (⊣) binds to a putative component (□) on the exposed surface of hepatocytes. We assume that anti-LPM Fabs 3–6 bind to □. When an MB6A cell binds at an interhepatocyte boundary, contact is made with an component on the hepatocyte exclusively present at the contiguous face (○), leading to infiltration of the MB6A cells. This infiltration can be inhibited with anti-LPM Fab 3, 4 and 5 (binding to ○). Anti-MB6A Fabs 1 and 2 do not bind to the putative component on the MB6A cell surface involved in infiltration (⊣●). The reasons for postulating the latter component are given in the Discussion.

then anti-MB6A Fab should have reduced both the interaction index and the infiltrated fraction. However, the latter parameter was not affected (Table 2). This indicates that on MB6A cells a second component is present also, mediating infiltration by interaction with the contiguous surface molecule. This second component is apparently not detected by our anti-MB6A antisera. In Fig. 1 a hypothetical model is presented for MB6A infiltration into hepatocyte cultures and the cell surface components involved. (The reversibility of adhesion to the exposed hepatocyte surface has been discussed elsewhere; Roos & Van de Pavert (1982).)

If it is assumed that lymphosarcoma cells have a higher affinity for the contiguous surface molecule, their propensity to accumulate between hepatocytes may be explained. When moving along hepatocyte surfaces, the tumour cells will have a

high tendency to move from the less adhesive exposed surface to the highly adhesive contiguous surface, whereas the reverse will occur much less frequently. This mechanism may account for the diffuse spread of these tumour cells in the intact liver (Dingemans, 1973; Roos *et al.* 1977; Roos, 1981).

The antisera used were multispecific and may also have been directed against surface components important for various cellular activities. It is conceivable that some of our observations are actually due to inhibition of such activities, that may be important for adhesion and infiltration. Viability of MB6A cells was, however, not affected as shown by trypan blue exclusion, and morphological signs of acute damage to hepatocytes or MB6A cells after treatment with the antibodies were not observed. More subtle influences of the antibodies leading to such indirect effects can, however, only be definitely excluded by the identification of the membrane components involved in the observed effects.

In order to exclude the possibility that binding of univalent antibodies to the cells is in itself responsible for the effects described, we used univalent anti-T200 antibodies directed against MB6A cells, and univalent antibodies against the asialoglycoprotein receptor on the hepatocytes, as described by Ashwell & Morell (1974). The antibodies bound to the respective cells, as shown by immunofluorescence, but did not affect the interaction index or infiltrated fraction.

The inhibiting anti-LPM and anti-MB6A antibodies could be absorbed out by freshly isolated hepatocytes and MB6A cells, respectively (Tables 3 and 6). It is not surprising that the inhibiting activity of 0·375 mg anti-MB6A Fab could be absorbed out with as few as 20×10^6 MB6A cells, and of 0·275 mg anti-LPM Fab with only 5×10^6 hepatocytes. Only a very small number of the Fab fragments may be directed against the relevant surface components, as is suggested by the fact that pretreatment of MB6A cells or hepatocytes with less Fab strongly reduces inhibition. Also other workers (Bertolotti *et al.* 1980; Öbrink, personal communication) found that only a small percentage of antibodies of multispecific antisera is directed against adhesion molecules.

The anti-MB6A antibodies did not cross-react with cultured rat hepatocytes, as shown by immunofluorescence and by the fact that treatment of 0·375 mg Fab fragments with 5×10^6 freshly isolated hepatocytes did not result in absorption of inhibiting antibodies. On the other hand, however, anti-liver antibodies did cross-react with MB6A cells, and we could not establish unequivocally whether MB6A cells could absorb out inhibiting anti-liver antibodies.

Therefore, at present the possibility cannot be completely excluded that the anti-LPM antibodies may exert an effect in binding to the MB6A cells. This should be clarified by the identification of the molecule involved.

We gratefully thank Dr B. Öbrink and Dr G. Ashwell for the kind gift of some of the antisera used. We thank C. A. Feltkamp and D. A. M. Mesland for stimulating discussions, and M.A. van Halem for typing the manuscript. O.P.M. was supported by the Koningin Wilhelmina Fonds, The Netherlands Cancer Foundation, project no. NKI 80–6.

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(*Received* 21 *July* 1981)